APPENDIX F

Differential Frequencies of $p16^{INK4a}$ Promoter Hypermethylation, *p53* Mutation, and K-*ras* Mutation in Exfoliative Material Mark the Development of Lung Cancer in Symptomatic Chronic Smokers

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Purpose: The aim of this study was to investigate the frequency of three (epi)genetic alterations (*p53* and K-*ras* mutations and $p16^{INK4a}$ promoter hypermethylation) in symptomatic chronic smokers compared with patients with lung cancer and to evaluate the use of exfoliative material for such analyses.

Patients and Methods: Fifty-one patients with histologically confirmed lung cancer and 25 chronic smokers (> 20 pack-years) were investigated for mutations in the K-*ras* (codon 12) and *p53* (codons 248, 249, and 273) genes and for allelic hypermethylation of the $p16^{INK4a}$ gene. DNA was isolated from sputum and bilateral bronchial lavage, and brushings were taken at bronchoscopy.

Results: Forty-one genetic lesions were detected within exfoliative material from the group of 51 patients with lung cancer and 10 lesions in the chronic smoker group. K-*ras* mutations occurred exclusively in the lung cancer group, whereas *p53* mutations and $p16^{INK4a}$ promoter hypermethylation were also found in chronic smokers. Three of eight chronic smokers who harbored an (epi)genetic alteration were subsequently diagnosed with lung cancer. Analysis of sputum yielded information equivalent to that of samples obtained during bronchoscopy.

Conclusion: $p16^{INK4a}$ promoter hypermethylation and *p53* mutations can occur in chronic smokers before any clinical evidence of neoplasia and may be indicative of an increased risk of developing lung cancer or of early disease. K-*ras* mutations occur exclusively in the presence of clinically detectable neoplastic transformation. Molecular analysis of sputum for such markers may provide an effective means of screening chronic smokers to enable earlier detection and therapeutic intervention of lung cancer.

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THE ACCUMULATION of genetic damage is a hallmark in the development of lung cancer, a tumor with growing incidence and the leading cause of cancer-related deaths in industrial nations. The vast majority of lung cancers are smoking-related, whereby the number of genetic lesions present correlates with the intensity of carcinogen inhalation. Despite intensive clinical research, there has been no significant improvement in the therapy of lung cancer during the past 10 years. This is largely a result of the early metastatic spread of such tumors relative to the time of clinical diagnosis. Using the current standard methods of diagnosis and treatment, fewer than 15% of patients with lung cancer will survive their disease.[1] The development of efficient diagnostic methods to enable earlier detection and therapeutic intervention in lung cancer is clearly important.

In one recent study, screening using cytologic examination of sputum identified a group of patients with preinvasive and microinvasive lung cancer, who demonstrated high survival rates after surgical removal or localized therapy.[2] In other studies, however, annual cytologic sputum examination combined with chest radiography did not improve the overall survival of patients,[3,4] probably because microscopic metastatic disease had already occurred.

Significant progress has now been made in the understanding of the genetic basis of lung cancer,[5,6] which raises the hope that molecular markers could be used to detect lung cancer earlier in its natural history. To date, molecular analyses of sputum or bronchial lavage fluid have concentrated on mutations in oncogenes, such as K-*ras*, or tumor suppressor genes, such as *p53*, as potential markers of lung cancer[7] (reviews[8-11]). Mutations in K-*ras* are found in non–small-cell lung cancer (NSCLC), predominantly in adenocarcinoma, and the rate ranges between 15% and 50%, depending on the study material and the sensitivity of the assay used.[12-18] The vast majority of K-*ras* mutations affect codon 12 (> 90%).[19] Mutations of the *p53* gene are detectable in 50% to 70% of patients with lung cancer and are found in all histologic types.[20-24] These mutations

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Table 1. Frequency of *p53* (Codon 248, 249, and 273) and K-*ras* (Codon 12) Mutations and $p16^{INK4a}$ Promoter Hypermethylation Status in Exfoliative Material (Sputum, Bronchial Lavage, and Brushings) From Patients With Histologically Confirmed Lung Cancer and From Symptomatic Chronic Smokers

	Total No. of Patients	*p53* Mutation		K-ras Mutation		$p16^{INK4a}$ Hypermethylation		Any Lesion	
		No.	%	No.	%	No.	%	No.	%
Total patients with tumors	51	7	14	8	16	26	51	35	69
Tumor cell type									
NSCLC	31	4	13	8	26	18	58	25	81
Squamous cell	22	3	14	4	18	13	59	17	77
Adenocarcinoma	5	0		1	20	3	60	4	80
Large cell	2	1	50	2	100	1	50	2	100
Other	2	0		1	50	1	50	2	100
SCLC	20	3	15	0		8	40	10	50
Chronic smokers	25	3	12	0		7	28	8	32

comprise both allelic loss and point mutations, the latter clustering within the hot spot regions of the *p53* gene.[25] Although K-*ras* mutations seem to occur late in lung cancer tumorigenesis,[26] somatic alterations of the *p53* gene are found in different tumor stages and may even occur in metaplastic and dysplastic states.[27-29] More recently, inactivation of the $p16^{INK4a}$ tumor suppressor gene has been shown to occur commonly in lung cancer,[30-32] resulting from either allelic loss or mutation of $p16^{INK4a}$ or regional hypermethylation of CpG islands in its promoter region.[33] In contrast, $p16^{INK4a}$ promoter hypermethylation is detectable neither in normal lung tissue nor in a variety of other types of non-neoplastic tissue investigated.[30,34] Little is known, however, about the sequence of these various events in the development of lung cancer.

In the present study, we examined exfoliative material (sputum, bronchial lavage fluid and brush cytology) from 51 patients with lung cancer and 25 chronic smokers (> 20 pack-years and suffering from benign lung disease) for the prevalence of CpG-island hypermethylation of $p16^{INK4a}$ and mutations in the K-*ras* and *p53* genes. Our aim was to establish the frequency of each of these genetic or epigenetic lesions in chronic smokers compared with patients with established lung cancer and, thereby, to investigate their potential benefit as molecular markers of preneoplastic or early disease. In particular, we addressed the question of whether such genetic and epigenetic changes can be detected in easily obtainable exfoliative material such as sputum, which would render such analyses more suitable for a screening program.

PATIENTS AND METHODS

Patient Selection

All diagnostic bronchoscopies were performed in the Department of Respiratory Medicine in the Krankenhaus Neukölln in Berlin between February 1996 and February 1999 by one of the authors (W.P.). Patients were selected at random during the author's (W.P.) duty periods in the Endoscopy Unit. Patients were selected for the lung cancer group if radiography was suggestive of an endoscopically visible tumor. Symptomatic chronic smokers were included in the study only when chest radiography was not indicative of tumor or chronic inflammatory disease such as sarcoidosis. All were heavy smokers of greater than 20 pack-years. All subjects gave written informed consent before the investigation.

Induced sputum was obtained during local anesthesia using 10 to 15 mL of nebulized lignocaine. Flexible fiberoptic bronchoscopy was performed using an Olympus fiberoptic bronchoscope (Olympus Optical Co, Hamburg, Germany) via the oral route. Intrabronchial procedures were performed in the following sequence: bronchial lavage with 10 mL of sterile saline, brushings from the main bronchus or tumor site, then biopsy of the tumor, bilaterally, starting with the nontumor side. In the chronic smokers, lavage and brushings were performed in the main bronchi in each lung and one to two bronchial biopsies were taken from one side. Brushings were suspended in sterile saline. Biopsies were fixed in 4% formalin. Sputum and bronchial lavage specimens were each mixed with 100 mg of acetylcysteine for 10 minutes to break down mucus, before passing the fluid through several layers of sterile gauze. After centrifugation (2000 rpm for 5 minutes at 4°C), the cell pellet was washed once in phosphate-buffered saline. Cell pellets were stored in microtubes at −20°C.

The mean age of the lung cancer patient group was 63 years (range, 46 to 84 years; median, 62 years) and included male (n = 39) and female subjects (n = 12). The mean age of the symptomatic chronic smoker group was 56 years (range, 38 to 79 years; median, 54 years) and included male (n = 22) and female subjects (n = 3).

Patients were clinically staged by the author (W.P.), and tumor biopsies were independently graded by two pathologists. Of 51 patients with lung cancer, 20 had small-cell lung cancer (SCLC) and 31 had NSCLC; histologies are listed in Table 1.

Table 2 shows the clinical staging of the patients selected for the study. Five patients had stage I disease, four had stage II disease, 20 had stage III disease, and 22 had stage IV disease, according to the revised staging system.[35]

Tumor Biopsies

DNA from paraffin-embedded tumor biopsy tissue was extracted from 10-μm sections according to "Method A," as described by Frank et al.[36] In parallel, sections were hematoxylin and eosin–stained and pathologically reviewed to ensure inclusion of tumor tissue.

Table 2. Frequency of Each Genetic Lesion Detected in Relation to the Tumor Staging

		p53 Mutation		K-*ras* Mutation		$p16^{INK4a}$ Hypermethylation	
	Total No.	No.	%	No.	%	No.	%
Pathologic stage							
IB	5	0		0		3	60
IIB	4	0		1	25	4	100
IIIA	6	1	17	0		4	67
IIIB	14	0		3	21	5	36
IV	22	6	27	4	18	10	45

Sputum and Bronchial Lavage Samples

DNA was extracted from 50% of homogenized pellets of sputum, bronchial lavage fluid (BAL), or brushings, using a Qiagen Tissue Kit (Qiagen, Hilden, Germany) for DNA preparation, according to the suppliers' specifications. Each DNA sample was tested for the presence of genomic $p16^{INK4a}$ alleles by polymerase chain reaction (PCR) using primer pair p16-W.[37]

p53 *Mutation and K-*ras *Mutation Analysis*

Mutation analysis was performed as previously described.[7] Briefly, 50 to 200 ng of DNA were preamplified for 30 cycles in a volume of 50 μL containing 1 U of eLONGase (GibcoBRL, Life Technologies, Rockville, MD). Purified PCR products were diluted 1:30 and subjected to the following PCR–restriction fragment length polymorphism analysis. Two microliters of the diluted PCR products was reamplified with mismatch primers and digested with appropriate endonucleases. Codons 248, 249, and 273 were examined for *p53* mutations and codon 12 for K-*ras* mutation. Primer sequences and reaction conditions have been described elsewhere.[7] A patient was considered to be a carrier of a mutation when at least one sample (sputum, side-specific BAL or side-specific brushings) revealed a clear positive result in PCR analysis.

$p16^{INK4a}$ *Hypermethylation Assay*

Bisulfite conversion. Existing bisulfite conversion protocols[37-39] were modified for use with this patient material. Briefly, 10% of prepared DNA (10 to 200 ng) and 2 μg of Poly(dA-dT)Poly(dA-dT) copolymers (Amersham Pharmacia Biotech, Bucks, United Kingdom) were denatured for 20 minutes at 42°C by adding 1 N NaOH in a volume of 50 μL (to a final concentration of 0.3 mol/L). Fresh solutions of 10 mmol/L hydroquinone (Sigma Chemical Co, St Louis, MO; 30 μL) and 3 mol/L sodium bisulfite, pH 5.0 (Sigma Chemical Co; 520 μL) were added and the solution was gently mixed, overlayed with mineral oil, and incubated in the dark for 12 to 13 hours at 50°C. The aqueous phase was recovered using the Wizard DNA Clean-up System (Promega, Madison, WI). The elution efficiency of small DNA quantities was significantly improved by successive elution of bound DNA using prewarmed (80°C) TE buffer, pH 7.6 (50 μL of TE, 15 minutes incubation; 30 μL TE, 1 minute; centrifugation at 9,000 rpm for 20 seconds). The purified DNA was subsequently mixed with 1 mol/L NaOH to a final concentration of 0.3 mol/L and incubated for 20 minutes at 37°C to ensure complete desulfonization. DNA was ethanol-precipitated in the presence of 10% volume sodium acetate, and the resulting pellet, after washing with 70% ethanol, was resuspended in 50 μL of H_2O and stored at −20°C.

Primer extension preamplification (PEP). The genomic amplification was performed following a protocol by Zhang et al.[40] Twenty microliters of bisulfite-treated DNA was added to 5 μL of a 400-μmol/L solution of gel-filtrated N15 primers (TIB MOLBIOL, Berlin, Germany), 0.6 μL of dNTPs (each at 20 mmol/L), 6 μL of 10× PCR buffer, and 5 U of *Taq* polymerase (Qiagen) and H_2O (DuPont Merck Pharmaceutical Company, Wilmington, DE) to yield a total volume of 60 μL (whereby the quality of random primer synthesis proved to be of special importance). After initial denaturation at 94°C for 3 minutes, 50 primer extension cycles were performed in a Perkin Elmer 9,600 thermocycler (Perkin Elmer, Norwalk, CT) using the following parameters: 1-minute denaturation at 92°C, 2-minute annealing at 37°C, followed by a 3-minute linear ramping to 55°C, and 4-minute elongation at 55°C. Protocols with a faster ramping rate or stepwise temperature progression resulted in inefficient PEP amplification.

Semi-nested methyl-specific PCR (MSP). The detection of hypermethylated $p16^{INK4a}$ alleles was facilitated using a semi-nested PCR protocol in a 25-μL reaction volume. In the first PCR, a 5-μL aliquot of the PEP product was added to 200 μmol/L dNTPs, 0.4 μmol/L each primer (p16Mf: 5′-TTA TTA GAG GGT GGG GCG GAT CG; p16M2r 5′-CCA CCT AAA TCG ACC TCC GAC CG), 1× PCR buffer (Qiagen) and 0.65 U of *Taq* Polymerase. The second PCR contained 5 μL of the 1:10 diluted product of the first PCR, 200 μmol/L of dNTPs, 0.4 μmol/L of each primer (p16Mf, p16Mr 5′-GAC CCC GAA CCG CGA CCG TAA), 1× PCR buffer (Qiagen) and 0.65 U of *Taq* Polymerase. Both reactions comprised 30 cycles with 95°C for 30 seconds, 65°C for 30 seconds, 72°C for 40 seconds, with initial denaturation at 95°C for 3 minutes and a final elongation at 72°C for 10 minutes.

To control for complete bisulfite conversion and subsequent PEP amplification, a semi-nested PCR was also performed for the nonmethylated alleles. The same protocol, including 1× solution Q (Qiagen) to optimize buffer conditions, was performed using primers p16Uf (5′-TTATTA GAGGG TGGGG TGGAT TG) and p16U2r (5′-CCACC TAAAT CAACC TCCAA C) in the first reaction and primers p16Uf and p16Ur (5′-CAACC CCAAA CCACA ACCAT AA) in the second. The annealing temperature was lowered to 58°C.

Statistics

Statistical analysis of frequency distributions was evaluated by the χ^2 or Fisher's exact test, as appropriate, using the Statistical Package

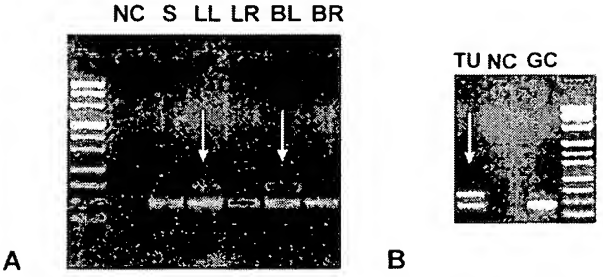

Fig 1. K-*ras* analysis. (A) Sputum, bronchial lavage left and right, brushings left and right, negative control, demonstrating mutations in lavage and brushings from the left side (adenocarcinoma left lobe). (B) Mutation confirmed in tumor biopsy. Abbreviations: S, sputum; LL, lavage left; LR, lavage right; BL, brushings left; BR, brushings right; NC, negative control; TU, tumor biopsy; GC, genomic DNA.

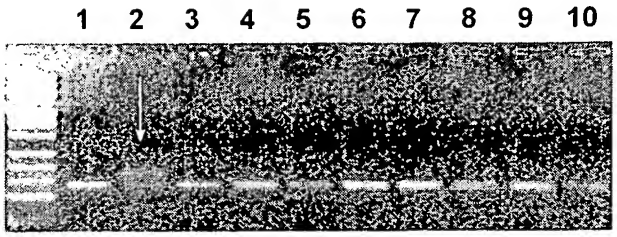

Fig 2. Example of *p53* (codon 273) restriction fragment length polymorphism analysis of bronchial lavage from patients with lung cancer. The arrow in lane 2 indicates a mutation.

for Social Science (SPSS, Inc, Chicago, IL). Statistical significance was taken as $P < .05$.

RESULTS

p53 mutations (codon 248, 249, or 273) were detected in seven samples (14%) from patients with tumors, of whom three had squamous cell carcinoma (14%; n = 22), one had large-cell carcinoma (50%; n = 2), and three had SCLC (12%; n = 20) (Table 1). Mutation in the *p53* gene was also detected in three samples (12%) from chronic smokers at the time of bronchoscopy. There was no statistically significant difference between patients with lung cancer and chronic smokers in the frequency of *p53* mutation (P = .572).

Mutations in the K-*ras* gene (codon 12) were found in eight samples (16%) from patients with confirmed lung cancer, including four patients with squamous cell carcinoma (18%; n = 22), one with adenocarcinoma (33%; n = 3), and two with large-cell carcinoma (100%; n = 2) (Table 1). No K-*ras* mutation was found in any patient with SCLC or in any of the chronic smokers. There was a statistically significant difference between patients with lung cancer and chronic smokers in the frequency of K-*ras* mutation (P = .034). Figures 1 to 3 show examples of samples investigated for K-*ras* and *p53* mutations and $p16^{INK4a}$ hypermethylation.

Modification of the standard MSP protocol[37,41] was necessary to analyze the exfoliative material for $p16^{INK4a}$ methylation status (see Methods). This was achieved by inserting a genomic amplification step, as described by Zhang et al,[40] before performing a semi-nested, rather than

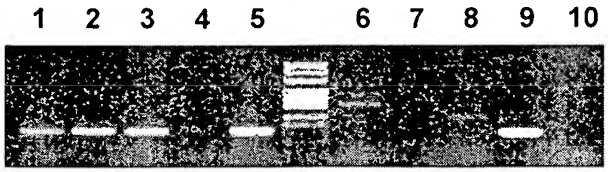

Fig 3. $p16^{INK4a}$ promotor hypermethylation analysis of sputum-DNA from lung cancer patients. Lanes 1 to 3, 5, and 9 represent hypermethylated alleles.

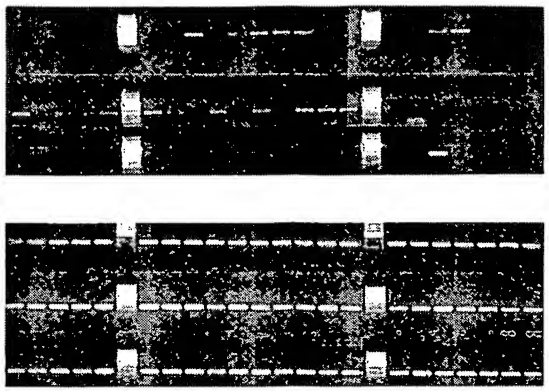

Fig 4. Control of sputum-derived DNA for bisulfite conversion with primers specific for nonmethylated sequences. (A) Conventional methyl-specific PCR (MSP) and (B) modified protocol with genomic amplification (PEP) and subsequent semi-nested PCR (see Patients and Methods).

a conventional, MSP analysis. Figure 4 illustrates the gain in sensitivity achieved by the modified MSP protocol (Fig 4A) compared with the conventional protocol (Fig 4B).

Hypermethylation of the $p16^{INK4a}$ promoter was found in 26 patients with lung cancer (51%; n = 51) and in seven chronic smokers (28%; n = 25), with a statistically significant difference between the two groups (P = .048). Those tumor patients who displayed hypermethylation of the $p16^{INK4a}$ promoter comprised 13 with squamous cell carcinoma (59%; n = 22), three with adenocarcinoma (60%; n = 5), one with large-cell carcinoma (50%; n = 2), one with undifferentiated NSCLC (50%; n = 2), and eight with SCLC (40%; n = 20) (Table 1).

The size of the tumor at the time of bronchoscopy had a clear effect on our ability to detect *p53* and K-*ras* mutations (Table 2). Of 15 patients with limited disease (stages I to IIIA), only one K-*ras* mutation (in a patient with stage II disease) and one *p53* lesion (in a patient with stage IIIA disease) were found. In these two cases, both tumors were larger than 3 cm. In contrast, epigenetic modification of $p16^{INK4a}$ was detected in 11 samples (73%; n = 15) from patients with tumors at or below tumor stage IIIA, as well as in 15 (42%) of 36 patients with stage IIIB or IV disease.

Table 3 shows a comparison of the number of lesions found in each of the types of exfoliative material analyzed. The frequency of genetic lesions detected in sputum samples was comparable to that detected in either BAL or brushings taken during bronchoscopy. This applied to all three genetic lesions analyzed in patients with lung cancer as well as chronic smokers. Notably, the frequency of $p16^{INK4a}$ promoter hypermethylation detected in both patients with lung cancer and chronic smokers was highest in sputum.

Table 3. Frequency of Genetic Lesions Detectable in Different Types of Exfoliative Material for the Chronic Smoker and Patients With Lung Cancer Groups

	Patients With Tumors (n = 51)		Chronic Smokers (n = 25)	
	No.	%	No.	%
Sputum				
K-*ras* mutation	5	10	0	
p53 mutation	4	8	1	4
$p16^{INK4a}$ hypermethylation	18	35	4	16
BAL				
K-*ras* mutation	6	12	0	
p53 mutation	4	8	2	8
$p16^{INK4a}$ hypermethylation	11	22	3	12
Brushings				
K-*ras* mutation	4	8	0	
p53 mutation	5	10	0	
$p16^{INK4a}$ hypermethylation	8	16	2	8

To investigate whether the three genetic lesions occurred in combination in either of our patient groups, we analyzed the total number of events found in each patient (Table 4). One of the tumor patients, but none of the chronic smokers, bore all three genetic alterations. Two of the investigated genetic lesions were detected simultaneously in four tumor patients (8%) and two chronic smokers (8%). A single genetic lesion was detected in 30 tumor patients (59%) and six chronic smokers (24%).

The redundancy of genetic alterations throughout the different levels of material examined was analyzed to gain information about the expansion and dissemination of clonal cells that harbored the lesions. As summarized in Table 5, a given genetic lesion in the chronic smoker group was found exclusively at one level of the exfoliative material examined (sputum, BAL or brushings), with one exception, in which $p16^{INK4a}$ hypermethylation was detected in sputum and BAL. Clinical follow-up of this patient revealed the development of lung cancer after bronchoscopy. The same genetic lesion was detected at more than one level (sputum, BAL or brushings) in 17 tumor patients, of whom five demonstrated the alteration at all three levels.

Table 4. Synopsis of Genetic Lesions (K-*ras* and *p53* Mutations, $p16^{INK4a}$ Hypermethylation) Detected in Exfoliative Material From Patients With Tumors and Chronic Smokers

	No. of Mutations					
	3		2		1	
	No. of Patients	%	No. of Patients	%	No. of Patients	%
Patients with tumors (n = 51)	1	2	4	8	30	59
Chronic smokers (n = 25)	0		2	8	6	24

To establish whether the genetic lesions identified within the exfoliative material could be attributed to the tumor itself, DNA was extracted from paraffin-embedded biopsies from 21 patients with tumors and three chronic smokers (numbers were limited by the unavailability of biopsies or inadequate DNA quality) and analyzed for $p16^{INK4a}$ promoter hypermethylation and K-*ras* and *p53* mutations. Hypermethylation of the $p16^{INK4a}$ promoter could be confirmed within the tumor biopsy in four of the five cases in which it was detected within exfoliative material. K-*ras* and *p53* mutations were found in three of four and two of six tumors, respectively, for which corresponding exfoliative material gave a positive result. No genetic alterations were detected within any of the three biopsies obtained from chronic smokers, although *p53* mutation had been identified within exfoliative material from two of these cases. In no case did analysis of the biopsies of the tumor patients or chronic smokers reveal any genetic alteration that had not been identified within exfoliative material.

DISCUSSION

In this study, we compared the frequency of three genetic alterations (*p53* and K-*ras* mutations and $p16^{INK4a}$ promoter hypermethylation) in different types of exfoliative material obtained from chronic smokers and patients with lung cancer. Our results demonstrate a significant frequency of lesions (32% overall; Table 1) in chronic smokers of greater than 20 pack-years, who are considered to be at high risk of development of lung cancer. The absolute number of genetic alterations detectable, however, was significantly higher in patients with lung cancer than in chronic smokers (69% *v* 32% for any lesion). This finding is in line with the concept of field cancerization. Redundancy of events, whereby the same genetic lesion is detected at multiple sampling levels, was a feature of the lung cancer group, presumably because of clonal expansion.

Hypermethylation of the $p16^{INK4a}$ promoter was the most frequently detected alteration of the three markers investigated in exfoliative material, both in patients with lung cancer and in chronic smokers. The presence of this epigenetic lesion was also confirmed within the tumor material itself in the majority of cases examined, which indicates that $p16^{INK4a}$ promoter hypermethylation is not an incidental finding consequent to generalized epithelial damage, but rather results from clonal expansion of tumor cells. The frequency of $p16^{INK4a}$ promoter hypermethylation was higher in patients with confirmed lung cancer than in chronic smokers (35% and 16%, respectively, assayed in sputum), which suggests that it is not a prerequisite for neoplastic transformation in the lung but that, when it occurs, it does so as an early event. This is supported by the

Table 5. Frequency With Which a Genetic Lesion (K-*ras* and *p53* mutations, or $p16^{INK4a}$ Hypermethylation) Was Detected at One, Two, or Three Levels of Sampling (Sputum, BAL, Brushings) in Chronic Smokers or Patients With Lung Cancer

	Levels of Detection					
	3		2		1	
	No. of Patients	%	No. of Patients	%	No. of Patients	%
Patients with tumors (n = 51)						
K-*ras* mutation	1	2	5	10	2	4
p53 mutation	2	4	1	2	4	8
$p16^{INK4a}$ hypermethylation	2	4	6	12	18	35
Chronic smokers (n = 25)						
K-*ras* mutation	0		0		0	
p53 mutation	0		0		3	12
$p16^{INK4a}$ hypermethylation	0		1	4	6	24

finding that a high proportion of patients with early-stage (stages I to IIIA) lung cancer exhibited $p16^{INK4a}$ promoter hypermethylation (Table 2), with frequencies similar to those found by Ahrendt et al[31] in their analysis of bronchial lavage from patients with early-stage lung cancer. Our findings also corroborate and expand on the results from a recent study by Belinsky et al,[32] which investigated $p16^{INK4a}$ hypermethylation in sputum from a group of chronic smokers and seven patients with lung cancer and showed for the first time the presence of *p16* hypermethylation in individuals at risk of lung cancer. Our observations from a larger collective provide evidence that aberrant $p16^{INK4a}$ promoter hypermethylation accumulates during preneoplasia and early stages of lung cancer. Thus, it may constitute a candidate marker to enable detection of early lung cancer or even risk of disease in chronic smokers.

The presence of the investigated *p53* hot spot mutations did not differentiate between patients with tumors and chronic smokers (P = .572). Both these groups demonstrated a similar frequency of *p53* lesions (14% and 12%, respectively). In agreement with other investigators,[42,43] we did not find the presence of *p53* mutation to be correlated with any histologic subgroup. Interestingly, we could not confirm the presence of *p53* mutation detected in exfoliative material within corresponding tumor biopsy material in two thirds of cases investigated. This raises doubts that such a genetic alteration is attributable to the tumor itself. Instead, it is possible that *p53* mutation arises from incidental generalized epithelial damage. Such genetic alterations have been shown to occur within the bronchial epithelium of cancer-free chronic smokers and to be unrelated to any malignancy.[44] Thus, the specificity of *p53* mutation as a candidate marker of lung cancer is not optimal. Furthermore, despite analyzing three hot spot mutations in this study, only 20% to 25% of the *p53* mutations which are recognized to play a role in lung cancer can be expected to be detected with this protocol. This highlights the challenges of incorporating *p53* mutation analysis into a screening program for lung cancer.

The distribution of K-*ras* lesions differed from that of *p53* mutations and $p16^{INK4a}$ promoter hypermethylation. K-*ras* mutations in codon 12 were found exclusively in the tumor group and were restricted to NSCLC histologies (Table 1). This supports the findings of several other groups who have reported the association of K-*ras* mutation with lung cancer.[26,45] Our findings do not agree with those reports that demonstrate K-*ras* lesions in noncarcinogenic tissue of the lung.[46-49] This apparent discrepancy might arise from the use of differing types of patient material. Our investigation was performed on exfoliative material, and therefore, contamination with nontumor material might have influenced the sensitivity of our assays. Furthermore, although all studies applied DNA amplification protocols, the level of sensitivity is not necessarily comparable. For example, extremely sensitive enriched PCR protocols were applied in some studies, which allowed the detection of less than 1 allele in 1,000 normal alleles.[47,49] Perhaps these highly sensitive conditions account for the detection of K-*ras* mutations in nononcologic patients.[47,49] The relatively low frequency of K-*ras* mutation in lung cancer, however, precludes its use as a marker in isolation.

Although the sensitivity for any of the markers of lung cancer investigated in this study was relatively low, with frequencies ranging between 14% and 51% for the tumor group (Table 1), combining the three markers increased the detection rate to almost 70%. In line with our results, the use of four independent molecular markers was shown to increase the sensitivity, enabling detection of more than 90% of lung cancers (J. Herman, personal communication, April 1999). There is increasing evidence that clonal evolution of tumors from premalignant lesions is a complex process that involves multiple genetic abnormalities, which do not necessarily have a linear progression.[50] Thus, as our results intimate, effective detection of preneoplasia or early lung cancer is likely to mandate the use of a panel of molecular markers, spanning the various genetic alterations that might be present, rather than being feasible with single markers alone.

Analysis of tumor DNA did not reveal any lesion that had not been detected in exfoliative material. A further aim of this study was to evaluate the suitability of DNA obtained from sputum compared with that from bronchial lavage fluid or brushings for monitoring genetic and epigenetic alterations in the bronchial tree. Our results show that analysis of sputum, obtained by noninvasive means, offers a

comparable level of detection as samples taken by invasive bronchoscopy. In fact, a higher frequency of $p16^{INK4a}$ promoter hypermethylation was detected for chronic smokers, as well as patients with lung cancer, in sputum than in either bronchial lavage or brushings, which perhaps reflects the merits of screening a sample that is independent of anatomic location within the bronchial tree. Therefore, sputum could provide a valuable source of material for routine screening for lung cancer. Recently, abnormal DNA methylation was found in the serum of patients with lung cancer at all tumor stages at relatively high frequencies.[51] Because release of tumor-derived DNA into the circulation requires efficient tumor vascularization, however, screening of serum is unlikely to be able to detect preinvasive lesions early enough to improve the effects of therapeutic intervention. Thus, for the monitoring of individuals at high risk of lung cancer, sputum seems to be the best candidate material.

Finally, it is important to establish whether the genetic alterations investigated constitute markers for a risk of developing lung cancer or for established malignancy. We have approached this issue in two ways. As discussed above, DNA from tumor biopsies for approximately one half of our study collective was analyzed to investigate whether the genetic lesions detected in exfoliative material could be confirmed in and, thus, attributed to the tumor. This analysis demonstrated that the majority of K-*ras* and $p16^{INK4a}$ lesions found in exfoliative material of patients with lung cancer matched those found in primary tumor material. Because no such biopsy material was available for the chronic smokers, we observed their clinical history. Of eight patients with genetic lesions at the time of bronchoscopy, three cases subsequently developed a de novo cancer. Patient no. 028 developed lung cancer 1 year after bronchoscopy and is now deceased. Patient no. 052 developed cancer of the esophagus occluding the left main bronchus 14 months after bronchoscopy. Patient no. 078, who had a history of larynx carcinoma resected 12 years before, developed metastasizing lung cancer 1 year after bronchoscopy, of which he died. All three of these patients exhibited $p16^{INK4a}$ promoter hypermethylation at the time of bronchoscopy. In addition, patient no. 028 bore a *p53* mutation in codon 273. Although this evidence is circumstantial, it gives hope that detection of $p16^{INK4a}$ promoter hypermethylation in particular may be of value to the clinician in managing symptomatic chronic smokers. Such patients may warrant more regular follow-up with conventional diagnostic investigations. Clearly, prospective clinical studies are required to evaluate the potential benefit of such a marker in accelerating the diagnosis of lung cancer and thus maximizing the chances of effective therapeutic intervention.

In summary, this is the first study to compare detection of $p16^{INK4a}$ promoter hypermethylation and *p53* and K-*ras* mutations in collectives of symptomatic chronic smokers and patients with different stages of lung cancer and to evaluate the use of exfoliative material for such analyses. K-*ras* mutation was found exclusively in the lung cancer group and is thus a candidate marker for the presence of neoplastic transformation. Although *p53* mutation was detected in exfoliative material from both patients with lung cancer and symptomatic chronic smokers, its significance is unclear because this change was not necessarily attributable to the tumor material. We have shown that $p16^{INK4a}$ promoter hypermethylation occurs in chronic smokers in the absence of clinical evidence of neoplasia and that its frequency increases further in early stages of lung cancer. Therefore, hypermethylation of the $p16^{INK4a}$ promoter may constitute a marker of increased risk of developing lung cancer or of early disease.

This study has shown that analysis of sputum yields information equivalent to that obtained from bronchial lavage or brushings. Therefore, molecular analysis of sputum for a panel of markers including $p16^{INK4a}$ promoter hypermethylation and K-*ras* mutation may provide an effective means of screening symptomatic chronic smokers to enable earlier detection and therapeutic intervention of lung cancer.

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